The protein antigens secreted *in vivo* by adult male *Schistosoma mansoni*

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SUMMARY

Adult schistosomes can be transferred surgically from donor C57BL/6 mice to the portal veins of naive recipients with complete success. This procedure bypasses larval development and the antibody response of the host is directed against, and can be used to identify, those antigens released only by viable, mature parasites. Serum collected from the recipient mice (WTS) was used in Western blotting studies to probe fractionated parasite protein. Twelve immunodominant proteins were identified, ranging in molecular weight from 14 to 208 kDa. The magnitude of the IgG response against each antigen could be divided into 2 categories, on the basis of optical densitometry of the blots. In addition, defined parasite fractions were probed with WTS by Western blotting, in order to determine the relative abundance and distribution of each antigen in schistosome tissue. To confirm and expand on these initial observations, oligospecific polyclonal antibody for each immunogen was affinity purified from Western blots; it was then used in immunocytochemistry to identify the sources of secretion for 8 of the 12 antigens, at the cellular level. From the results, it appeared that after the transfer of adult worms, the first antibodies detected were mostly directed against the gastrodermis. At later times additional reactivity was expressed against the tegumental membrane. These differences probably reflect the relative abundances of the gut and tegumental secretory products.

Key words: *Schistosoma mansoni*, excretory/secretory (E/S) antigens, surgical transfer, *in vivo* culture, single-sex infection, antigen localization.

INTRODUCTION

Development of a vaccine against *Schistosoma mansoni* necessitates the investigation and detailed characterization of protective immune responses elicted by the parasite and identification of the relevant antigens involved. To this end, several workers have demonstrated that schistosome secretory antigens induce a protective, cell-mediated immune response in rats and primates (Auriault *et al*. 1984, 1985; Damonneville *et al*. 1986*b*). Active immunization of rats with schistosomula-released products (SRP) afforded between 46 and 83% resistance, whilst the passive transfer of anti-SRP serum conferred up to 83% protection on recipients (Damonneville *et al*. 1986*b*). More recently, interest has focused on the adult worm as a potential target of acquired immunity in man. Results from a clinical study by Dunne *et al*. (1992*a*, *b*) revealed a correlation between increased anti-adult worm IgE titres and resistance to reinfection with age. The IgE-reactivity appeared to be directed against a subset of adult schistosome proteins, with the specific response dominated by an antigen of M_r 22 kDa. Evidence suggests that the protective immune mechanism(s) in man and rat is dependent upon the establishment of a schistosome-specific IgE response (Capron *et al*. 1992). In view of this observation, the finding that elevated levels of reagins in rats were associated with adult worm E/S components, rather than parasite homogenates (Vannier *et al*. 1974; Pierce *et al*. 1983; Damonneville *et al*. 1986*a*), suggests that released components of living parasites could influence the expression of acquired immunity. Hence, the identification of excreted/secreted adult worm material could be of major importance in the search for potential vaccine candidates.

Adult worms are metabolically active, and release/secrete macromolecules from epithelial surfaces, such as the gut and tegument as part of their normal activities (Wilson & Barnes, 1979; Rotmans *et al*. 1981; Payares *et al*. 1985; Lewis & Strand, 1991). This propensity means that the mature schistosome represents a rich source of potential immunogens (Murrell, Vannier & Ahmed, 1974; Vannier *et al*. 1974; Rotmans *et al*. 1981; Pierce *et al*. 1983; Lewis & Strand, 1991). In view of this, the syncitial tegument is considered an important target for vaccine-induced immunological attack (Grzych *et al*. 1982; Kelly *et al*. 1985; Payares *et al*. 1985; Harn *et al*. 1987; Pearce & Sher, 1989). In addition, infected rodents exhibit an early and strong humoral response against a number of gut-associated antigens (Nash, 1978; Deelder *et al*. 1980; Chappell & Dresden, 1988). A large body of information is available about tegumental proteins and gut-associated products of

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adult worms, yet the full extent of antigen release by mature parasites has not been investigated comprehensively. Previous studies have described the difficulties of maintaining adult parasites successfully *in vitro* (Wilson & Barnes, 1974*b*). In addition, the low and inconsistent yield of material released into the medium by parasites, in conjunction with the variety of *in vitro* incubation procedures adopted by different workers (Murrell, Vannier & Ahmed, 1974; Lewis & Strand, 1991) have led to extensive but disparate analyses of released material. To date no study has characterized the proteins released *in vivo* by adult worms.

Problems associated with *in vitro* culture have led to the development of an *in vivo* culture system in mice, with which to identify the scarce secretory products of adult male worms (Saunders, Wilson & Coulson, 1987; Saunders *et al*. 1993). In these studies samples of ten 4-week-old male parasites, obtained from donor mice by portal perfusion, were introduced into the hepatic portal vein of naive recipients by surgical transfer, allowing the worms to function normally without the limitations of an *in vitro* environment. Worms recovered by portal perfusion from recipient mice at 8 weeks post-transfer were examined to ensure that they had not died, or incurred damage during transfer. It was concluded that *in vivo* culture excluded interference from larval and/or dead schistosome proteins, and that the male-only transfer circumvented the egg-induced pathology associated with disease, thereby permitting the extension of experimental study up to 8 weeks.

In this study, we report on the use of the surgical transfer technique to identify the products released by adult worms *in vivo*. Serum samples, taken from the recipient mice at weekly intervals post-transfer, were used to detect released material by Western blotting. Similarly, the major sites of antigen release were identified by probing soluble worm preparations, derived from different schistosome tissue, with worm transfer serum (WTS). The subcellular origin of each dominant antigen was resolved by the combination of antibody elution with immunocytochemistry of frozen worm sections. It is concluded that this system exhibits many advantages over conventional *in vitro* methods of secretory antigen identification.

MATERIALS AND METHODS

Parasites and hosts

A Puerto Rican strain of *S. mansoni* was maintained by routine passage through albino *Biomphalaria glabrata* and outbred LACA mice. C57BL/6 mice were infected with male cercariae, providing adult worms for male-only soluble worm antigen preparation (SWAP) and the worm transfer experiments.

Cercariae of one sex were obtained by exposing snails to single miracidia. The sex of the resultant cercariae was determined by polymerase chain reaction (PCR) following the method of Gasser, Morahan & Mitchell (1991). Snails shedding female, or male and female (mixed-sex) larvae were identified by the presence of a 500 base pair band and were discarded.

Surgical transfer of adult worms

The transfer of adult worms from donor C57BL/6 mice to recipient mice of the same strain was performed as described by Saunders *et al*. (1993). Four-week-old schistosomes were recovered from donors by portal perfusion with heparinized minimal essential medium (Gibco, Paisley, Scotland) containing 10 mM HEPES (MEMH) and 4 USP/ml of porcine heparin (Sigma, Poole, UK). Prior to transfer, the schistosomes were collected in a Petri dish and maintained, no longer than 45 min, in MEMH containing 10% normal mouse serum. Samples of 10 male worms were transferred into the superior mesenteric vein of naive recipients. Every 7 days following transfer, samples of blood were obtained from recipient mice by tail bleeding (60 μl), the serum separated, and centrifuged at 10000 ***g*** for 5 min before aliquotting and storage at -20 °C. After 8 weeks the mice were perfused to determine worm burden.

Antigen preparations

Total soluble preparation. SWAP was prepared using the procedure described by Ratcliffe & Wilson (1991). Female-only SWAP was derived from female worms which had been separated carefully from their male counterparts *in vitro*. To prevent protein degradation all antigen preparations were stored at -80 °C in Hank's Balanced Salt Solution (HBSS; Sigma) with protease inhibitors 1 (350 μg/ml pepstatin A, 250 μg/ml leupeptin, 20 mg/ml tosyl-1-lysine chloromethyl ketone dissolved in double distilled water) diluted 1:250, and 2 (350 mg/ml tosylamide-2-phenyl-ethylchloromethylketone, 8·7 mg/ml phenylmethylsulphonyl fluoride dissolved in ethanol) diluted 1:500. Protein content was determined by the method of Lowry *et al*. (1951) using bovine serum albumin (BSA) as the standard.

In vitro *culture supernatant*. Worms from mixed-sex infection were obtained as described above. They were resuspended in 20 ml of fresh MEMH, decanted into a sterile culture flask (Nunclon) and incubated for 3 h at 37 °C, 5% CO_2. Following culture the parasites were poured into Sterilin tubes and spun in a bench top centrifuge for 20 sec at 1000 ***g***. The parasite-free culture supernatant was removed and concentrated to a volume of 1 ml using a stirred

ultrafiltration cell (Amicon, Stonehouse, UK), as described by Harrop & Wilson (1993). The concentrated supernatant was stored at −80 °C with protease inhibitors 1 and 2. The worm bodies were washed 4 times with HBSS and stored under the same conditions. These male and female worms were subsequently used for a tegument membrane preparation.

Tegument membrane preparation. The tegument membrane was removed and purified according to the method of Roberts *et al*. (1983). As a final step the S2P pellet was resuspended in distilled water, centrifuged at 1000 ***g*** for 30 min, the supernatant removed and the pellet (S2P/Os), composed of purified tegument membrane, resuspended in Tris-buffered saline (TBS) and protease inhibitors.

Oesophageal, gonad and gut preparations. Male worms were washed 4 times with MEMH (without heparin) and poured into a Petri dish. They were carefully sectioned into 3 portions, using a pair of watch-maker's tweezers and iris scissors. The male worm oesophageal preparation was derived from tissue lying forward of the ventral sucker. A male gonad preparation was derived from material containing the testes; the germinal tissue lay posterior to the ventral sucker but within the anterior third of the worm body. The remainder of the worm tissue lying behind the gonads, was used to provide a preparation depleted in oesophageal and gonad material.

Approximately 500 male worms were dissected in total. The different tissues were placed in HBSS and stored in protease inhibitors at −80 °C. Upon thawing, all preparations were sonicated on ice for 5 min and the soluble fraction isolated, as described by Ratcliffe & Wilson (1991). Several preparations were made on different days. The soluble fractions were pooled for each tissue and concentrated as described earlier (Harrop & Wilson, 1993). The samples were stored at −80 °C with protease inhibitors.

Western blotting

Proteins were separated electrophoretically under reducing conditions by 1 dimensional SDS–PAGE according to the method of Laemmli (1970). Male SWAP was loaded at 80 μg of protein/1 cm of trough comb. Resolved fractions were electroblotted onto PVDF membrane (Millipore, Watford, UK; catalogue number IPVH 151 50), using procedures based on those of Towbin, Staehelin & Gordon (1979). The non-specific sites on the membrane were bound with blocking buffer, 2% BSA, 4% normal goat serum, 0·3% Tween 20 in PBS (150 mM NaCl, 1·5 mM KH_2PO_4, 8 mM Na_2HPO_4, 2·6 mM KCl) for 1 h at room temperature. The blotting procedure followed the protocol described previously by Roberts *et al*. (1987). Briefly, 5 mm strips of membrane were cut, sealed in plastic bags and left incubating with 2 ml of each test serum, diluted 1:1000 in blocking buffer, on a rotating windmill for 16 h at 4 °C. Normal mouse serum (NMS), and chronic mouse serum (CMS) obtained from mice infected with male cercariae for 12 weeks, were used as negative and positive controls, respectively. Both control sera were diluted 1:1000 with blocking buffer. After 5 washes in blot wash buffer (150 mM NaCl, 1·5 mM KH_2PO_4, 8 mM Na_2HPO_4, 2·6 mM KCl, 0·3% (v/v) Tween 20) over 45 min, each strip was incubated for 1·5 h at room temperature with the secondary antibody, goat anti-mouse IgG (F'ab specific) peroxidase conjugate (Sigma), diluted 1:2000. The strips were washed as before, and finally developed with diaminobenzidine (DAB) substrate (Sigma). The developed strips showing the proteins recognized by the worm transfer serum (WTS) over the 8 week experimental period were scanned by an optical densitometer (Bio-Rad, Hemel Hempstead, UK). The change in intensity of each band with time was calculated from the arbitary values obtained from the densitometry readings. From these figures the kinetics of the antibody response against each of the antigens over the 8 week experimental period could be determined.

In order to determine the primary sources of protein release, a selection of antigen preparations: oesophageal, gonad, posterior body, purified tegumental membrane, culture supernatant, female-derived SWAP and male-only SWAP, were loaded at equal protein concentrations (20 μg/well) on SDS–PAGE gels. The electrophoretic procedure was carried out as above. The fractionation patterns were visualized by staining the proteins with Coomassie blue solution.

To demonstrate the relative immunogenicity of the various preparations, the samples were electrophoresed by SDS–PAGE and then electroblotted onto PVDF membrane as described previously. The different preparations were probed with week 8 WTS (diluted 1:1000 with blocking buffer); all washes and incubations with primary and secondary serum were as described earlier. It was assumed that the antibody response to each immunogen would be enhanced by enriching for proteins in defined tissues. By visualizing and comparing the intensity of bands in the different preparations it was possible to assess the relative abundance of each antigen. Thus, these experiments gave a preliminary indication of the origin(s) of adult worm secretory antigens.

Antibody elution from Western blots

The location of the (E/S) proteins in the tissues of adult schistosomes was determined, using oligo-specific polyclonal antibody eluted from Western blots to probe parasite sections in immuno-fluorescence studies. Male SWAP was reduced and

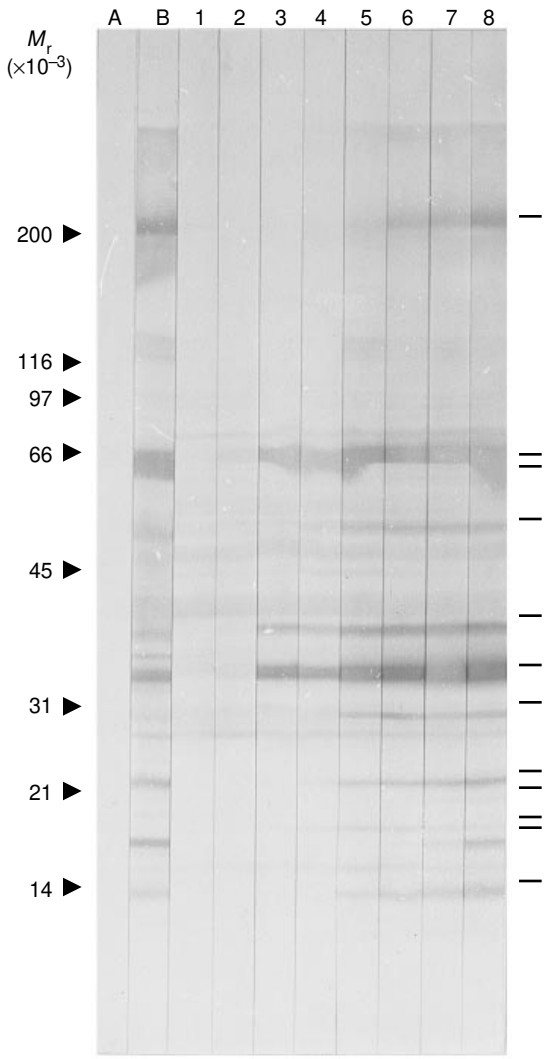

Fig. 1. Reactivity of the worm transfer serum (WTS) against fractionated adult male SWAP, as detected by Western blot analysis. Each lane was probed with the following sera (diluted 1:1000): (A) normal mouse serum (NMS), (B) chronic mouse serum (CMS), lanes 1–8 were incubated with WTS, weeks 1–8 respectively. The 12 immunodominant antigens are indicated by bars (see text for M_r).

loaded along the length of a trough comb at a concentration of 80 μg of protein/1 cm. The electrophoresis and transfer procedures are described above. The following antibody elution technique was modified from the 'antibody select' method described by Hall *et al*. (1984) and Coppell, Smith & Peterson (1993). Antigen-coated PVDF membrane was blocked and incubated with week 8 WTS diluted 1:1000 overnight at 4 °C. Two 5 mm strips were cut from either side of the blot, washed and developed with the secondary antibody and DAB substrate. The remainder of the blot was left incubating with the primary serum at 4 °C. After 5 washes the blot and strips were realigned and the dominant bands, as revealed by the adjacent strips, excised from the main body of the blot. Each band was cut into 1 mm pieces, placed in a 1·5 ml Eppendorf tube containing 400 μl of elution buffer, 200 mM glycine–HCl, pH 2·8, and agitated for 10 min. The samples were spun on a microfuge for 60 sec at 1000 ***g***, the supernatant removed and neutralized with 1 M Tris–HCl, pH 9·0. The specificity of the eluted antibody (diluted 1:25) for each of the dominant antigens was tested by reprobing a fresh blot.

Immunocytochemistry on parasite sections

The immunocytochemical procedure used in the present study was based on methodology developed by Riengrojpitak *et al*. (1989). Adult male *S. mansoni* worms were perfused from C57BL/6 mice, washed thoroughly in MEMH, embedded in OCT compound (Tissue-Tek, London, UK) and plunged into liquid nitrogen before storage at −80 °C. The tissue was sectioned (7 μm thickness) onto alcohol-cleaned slides at −28 °C using a cryostat (Slee, London, UK), air-dried and fixed for 1 min in absolute acetone. The slides were left to dry at room temperature for 30 min and stored at −20 °C.

Before staining, the sections were thawed for 30 min and fixed again in absolute acetone for a further 10 min. The fixed samples were washed 3 times for 30 min with PBS before treatment. Sections were blocked by incubating with undiluted normal goat serum for 60 min, and then the eluted antibody was applied undiluted. All incubations were carried out in a humid box, with the NMS and 8 week WTS (diluted 1:500 with blocking buffer) serving as negative and positive controls, respectively. The sections were probed overnight at 4 °C with the test and control samples. Following 3 washes in PBS and a further 10 min blocking step with normal goat serum, the secondary antibody, FITC-conjugated goat anti-mouse Ig (Nordic, Tilburg, The Netherlands) diluted 1:60, was applied and incubated in the dark. Finally, the samples were washed 3 times with normal goat serum and mounted in Citifluor (Agar Scientific, Stanstead, UK). The stained parasite sections were examined under a Nikon fluorescence microscope and photographed on Kodak Ektachrome, ASA 400.

RESULTS

Surgical transfer

Virtually all mice survived the surgery and remained healthy throughout the experiments. Provided that 10 live male worms were recovered intact at perfusion it was assumed that antibodies in the serum collected from the host were directed against male schistosome E/S products, and not somatic antigens leaked by dead or dying parasites.

The antibody response

The target molecules of antibody in WTS were detected by Western blotting of male-only SWAP.

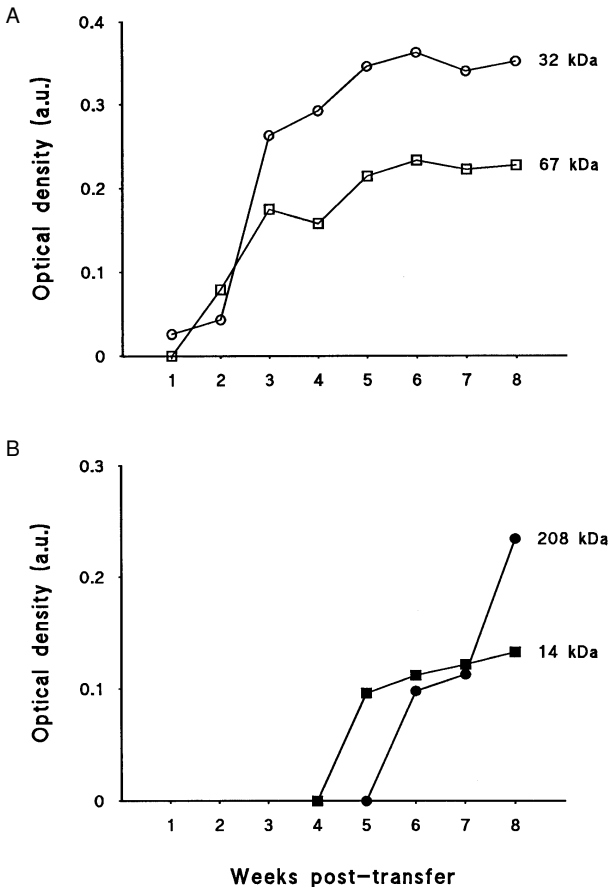

Fig. 2. The nature of the recipient host's IgG response, directed against individual E/S products, has been grouped into 2 categories dependent upon the intensity of antigen/antibody binding. (A) The kinetics of the IgG response against the 67 and 32 kDa molecules. The level of antibody rises rapidly following worm transfer and reaches a plateau 4 weeks later. This pattern is indicative of a category I response. (B) The kinetics of the IgG response against the 208 and 14 kDa antigens. Briefly, the antibody response is detected following a lag phase of 3–6 weeks, with IgG levels increasing slowly until termination of the experiment. This pattern is indicative of a category II response.

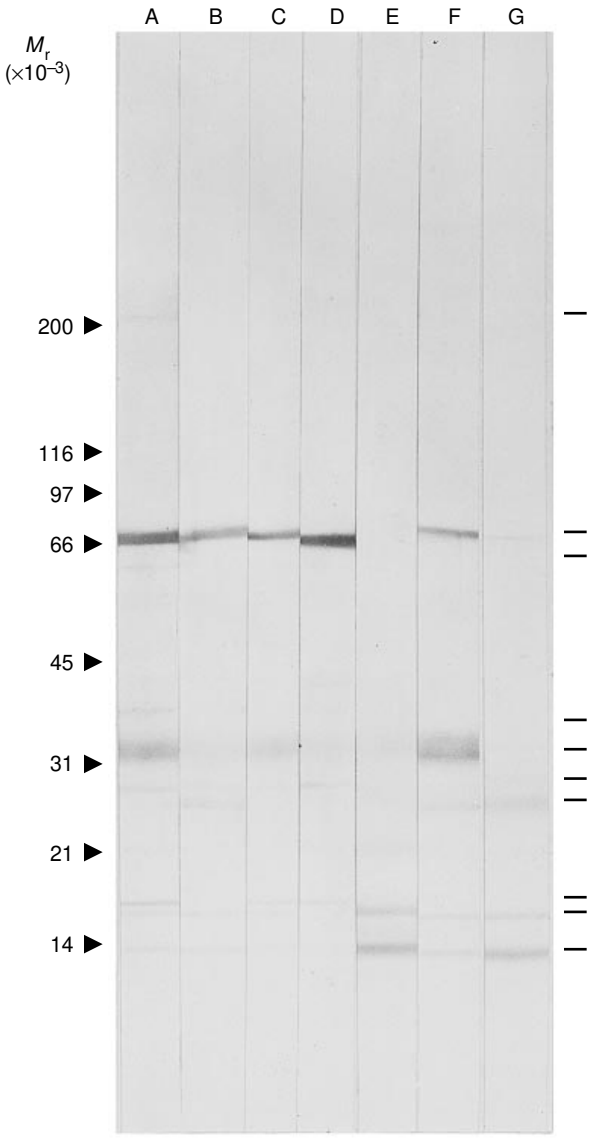

Fig. 3. Western blotting analysis of different antigen preparations probed with WTS week 8, diluted 1:1000. The antigen preparations were loaded at the same protein concentration (20 μg/well) in the following order. (A) Male-only SWAP, (B) soluble oesophageal fraction, (C) soluble gonad preparation, (D) soluble parasite material depleted of oesophageal and gonad components (rear), (E) isolated tegumental membrane fraction, (F) female-only SWAP, (G) adult worm *in vitro* culture supernatant. Antigens are shown by bars.

Twelve schistosome-specific antigens, M_r 208, 67, 62, 53, 38, 32, 30, 24, 22, 19, 18, and 14 kDa, are illustrated in Fig. 1. Upon visual analysis of the Western blot it appeared that the protein at 32 kDa possessed the same M_r as a gut protease described by Deelder, Reinders & Rotmans (1977). The similarity between the molecules was confirmed by probing fractionated SWAP with polyclonal antibody to the proposed haemoglobinase Sm32, kindly donated by Dr Rotmans, State University of Leiden, The Netherlands.

The immunogenicity of each antigen appeared to differ across the experimental course of 8 weeks with the lower molecular weight products detected later than the higher molecular weight products. Densitometry revealed that the antibody response to each band fell into one of two categories. Category one: the 67, 38, 32 and 22 kDa antigens were detected 1–2 weeks post-transfer. The antibody titre increased rapidly reaching a plateau 4 weeks later (Fig. 2). Category two: proteins at 208, 62, 53, 30, 24, 19, 18 and 14 kDa were detected following a lag phase of 3–6 weeks. The antibody titre increased slowly until termination of the experiment 8 weeks later (Fig. 2).

To demonstrate that there was no variability of response between animals, the transfer of worms was repeated. Following portal perfusion 8 weeks later 10 male worms were recovered intact from each of the mice. Qualitative analysis of WTS from all mice

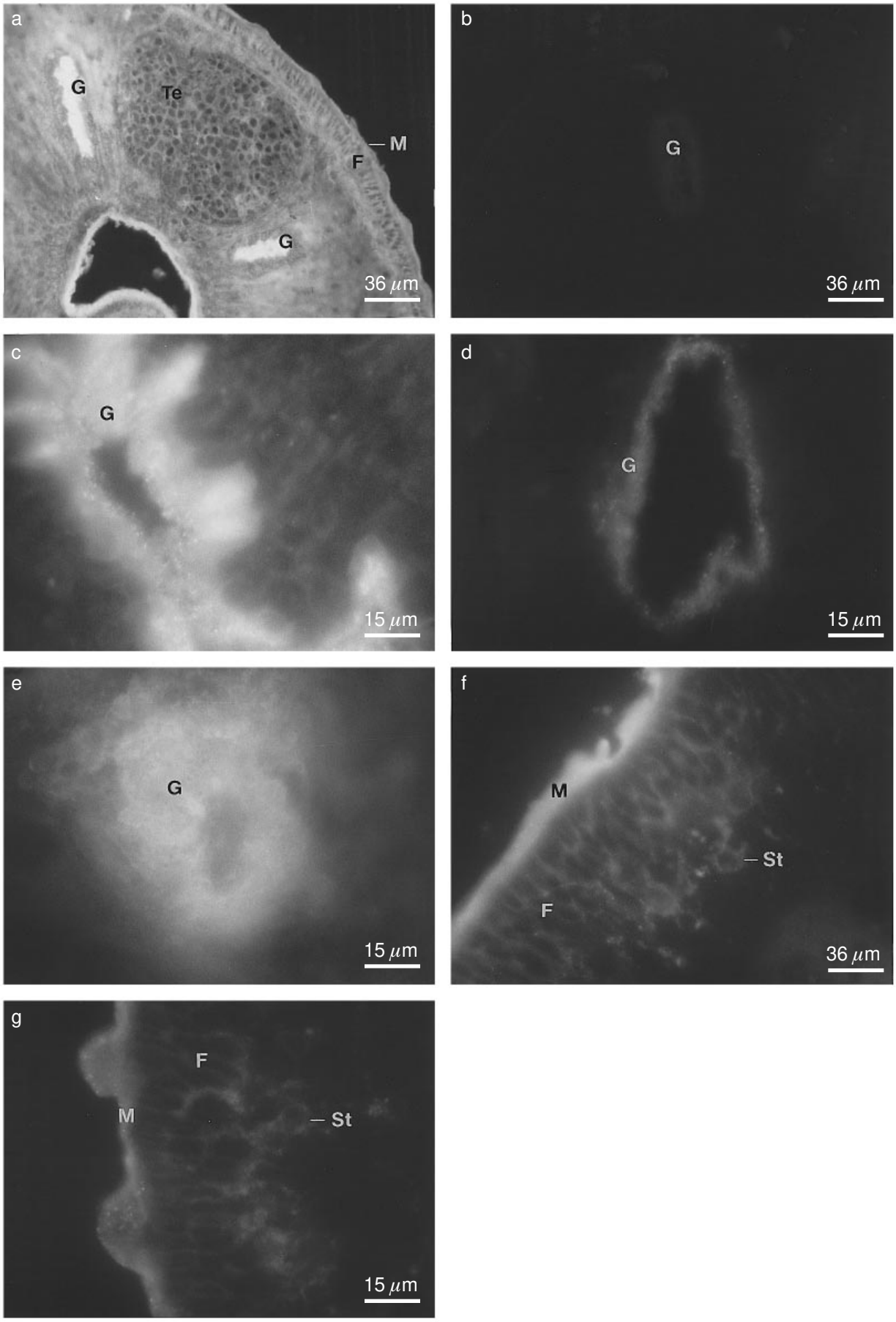

Fig. 4. For legend see opposite.

against male worm protein was carried out by Western blotting. The results indicated that the pattern of antigen recognition by antibody from each mouse was identical (data not shown).

Antigen abundance in parasite fractions

Coomassie-stained SDS–PAGE gels of the different antigen preparations. Soluble antigen preparations were loaded at equal concentrations on the same gel allowing qualitative comparison of the antibody response directed against each preparation. The pattern of fractionated proteins visualized on Coomassie-stained gels did not appear significantly different (data not shown). In general, there was a high level of homology between the banding patterns in each sample lane, with the exception of the culture supernatant and the tegumental membranes. These two protein preparations were less complex, containing fewer polypeptides (13 and 16 respectively) compared to male-only SWAP which comprised 30 distinguishable bands. The majority of the proteins were of higher molecular weights, with dominant bands at 65, 58, 47, 40 and 30 kDa. The 65 kDa molecule was present in much larger quantities in the culture fraction. In addition there was a heavily stained band at 12 kDa. The tegument membrane preparation contained a protein, molecular weight 22 kDa, which did not appear in the other samples.

Western blotting. After fractionation, the various protein preparations were electroblotted and incubated with the WTS, enabling the predominant sites of antigen release to be identified (Fig. 3). For example, it was assumed that a tegumental membrane antigen would be more abundant in a preparation enriched for tegumental membrane components. Hence, the IgG response against this antigen would be enhanced in the respective sample lane. The dominant 67 kDa protein was present in all preparations with the exception of the isolated tegumental membrane fraction. The 32 kDa antigen, of the same relative molecular weight as the proposed haemoglobinase, was prominent in the female-derived SWAP and male-only SWAP lanes. A previously unidentified 28 kDa antigen appeared in the oesophageal preparation and, to a lesser extent, in the female SWAP and culture supernatant. Lower molecular weight antigens 18 and 14 kDa were detected in the tegument membrane extract and culture supernatant. However, since it was necessary to equalize the protein content between preparations, the limited amount of material released during *in vitro* culture dictated the concentration used (approximately one quarter of that transferred in the initial Western blotting study). Thus, the overall reactivity of the immunoblot was reduced and the 53 and 24 kDa proteins were too scarce to bind detectable levels of antibody.

Tissue localization of released antigens

The above approach provided a simple method of locating the E/S products within parasite tissues; antibody elution together with immunocytochemistry proved a more refined technique. Oligospecific polyclonal antibody for 8 of the 12 dominant proteins was found to relocate faithfully to fresh blots, confirming its specificity. The eluted antibody was subsequently used to probe cryostat sections of parasite tissue.

The parasite sections probed with week 8 WTS as a positive control, were brightly stained (Fig. 4a). The tegument surface, gut, gonad and parenchymatous cells were strongly immunogenic. In direct contrast, the NMS did not appear to react with any worm structures (Fig. 4b). The samples of eluted antibody were grouped into those that bound either to the tegument and tegument membrane or to the gut of the worms. Antibody to the 67, 53, 38 and 32 kDa antigens demonstrated a high affinity for the parasite gut. Furthermore, it appeared that the distribution of these antigens within the cytoplasm of the cells differed, as distinguished by the patterns of fluorescence. The 67 and 53 kDa proteins were visible as tiny foci of fluorescence within the cell matrix (Fig. 4c–d). Under higher magnification the nuclei were clearly visible as regions of negative staining since they did not contain immunoreactive epitopes. The fluorescence observed when the parasite sections were probed with antibody to the 38 and 32 kDa proteins was less distinct (Fig. 4e). The eluted antibody bound intensely to the gastrodermis, and exhibited a similar pattern of staining to that observed with Rotmans' anti-32 kDa polyclonal antibody. The staining was diffuse and hence distinct foci of fluorescence were not demonstrable.

Antibody eluted from the lower molecular weight bands 24, 19, 18 and 14 kDa bound to the tegument of the schistosomes (Fig. 4f). The tegument membrane of the worm was bright and sharp with the contours of the tegumental pits clearly visible. Once more the pattern of binding was non-uniform with a gradation in fluorescence from the basal membrane

Fig. 4. Immunolocalization of secretory proteins within adult male schistosome tissue using affinity-purified antibody for each antigen of interest. Examples of cryostat sections probed with the following antibody are shown: (a) WTS week 8, positive control, (b) NMS, negative control, (c) eluted antibody against the 67 kDa antigen, (d) eluted antibody to the 53 kDa antigen, (e) affinity-purified antibody to the 32 kDa antigen, (f) eluted antibody to the 19 kDa antigen, (g) eluted antibody against the 14 kDa antigen. All sections were photographed under oil, and exposed for the same length of time. G, gut; F, muscle fibres; M, tegument, St, subtegumental cell; Te, male worm gonads.

to the apical membrane of the syncitial tegument. Beneath the basal membrane lay the circular and longitudinal muscles. Their surfaces were strongly stained creating a reticulum network of fluorescence. Subtegumental cells, embedded amongst the parenchymal cells, were evident at intervals along the dorsal surface of the worm when probed with the anti-14 kDa oligospecific polyclonal antibody (Fig. 4g). Under higher magnification it was difficult to determine if the 14 and 19 kDa antigens were localized at the surface of each subtegumental cell or present within the cell matrix.

DISCUSSION

The main objectives of this study were to identify, by molecular weight, the secretory proteins of adult schistosomes, and to locate their source(s) in worm tissues. For this purpose, the host's immune response provided the detection system to identify released macromolecules. The results reported here show that the mature male schistosome excretes/secretes 12 immunodominant macromolecules, ranging in M_r from 14 to 208 kDa. Western blots of the different antigen preparations probed with WTS revealed that the lower M_r proteins, which appeared late post-transfer, were associated with the schistosome tegument. This observation was verified by incubating oligospecific polyclonal antibody for each fraction with frozen parasite sections. Using this refined immunocytochemical technique it was possible to identify the sources of antigen excretion/secretion for 8 of the 12 immunogens. Four were immunolocalized to the gastrodermis, whilst the remainder were associated with the tegument membrane.

Prior to this study, workers have performed the surgical transfer technique using monkeys (Smithers, Terry & Hockley, 1969), mice (Boyer, Ketchum & Palmer, 1976; Saunders *et al*. 1993) and hamsters (Cioli, 1976; Saunders *et al*. 1987) as recipient hosts. However, it has not been used previously for the unequivocal identification of secretory antigens. The 12 antigens detected are a minimum estimate, since it is possible that hepatic Kuppfer cells could clear some schistosome-released proteins so effectively that they fail to prime the immune system. Phagocytosis and enzymatic degradation of released products by these cells would not only reduce the amount of antigen available for immune processing, but may also alter the character of the native protein, reducing it to a series of smaller immunogenic peptide units. Furthermore, it is possible that some secreted proteins could be poorly immunogenic in the C57BL/6 mouse strain.

The magnitude of the IgG response against each antigen could be divided into 2 predominant categories. The first encompassed proteins of M_r 67, 53, 38 and 32 kDa, identified 1–2 weeks post-transfer. The second, comprising most of the lower molecular weight proteins, induced a later IgG response. Possible explanations for the disparity between antibody responses may be (a) differential processing of proteins by antigen-presenting cells, (b) differences in the immunogenicity of epitopes, (c) the relative abundance of released material or (d) differential clearance of schistosome molecules in the liver.

Previous investigations have adopted different *in vitro* approaches to detect the release of secretory products. Despite inherent disadvantages associated with *in vitro* culture Atkinson & Atkinson (1982) succeeded in maintaining radio-isotope labelled adult parasites in serum-supplemented medium for up to 2 weeks; subsequent fluorographic analysis revealed that 74 polypeptides were commonly released by male and female worms. Lewis & Strand (1991) performed a similar investigation, but immunoprecipitated the fractions with schistosome-infected human serum and then resolved the antigens by 2D SDS–PAGE. These workers detected an extensive range of antigens from 220 to 20 kDa; 8 of the immunoprecipitated glycoproteins identified were greater than 60 kDa. In contrast, the present study revealed only 3 higher M_r proteins (208, 67, 62 kDa) eliciting an antibody response. It is also noteworthy that the study by Lewis & Strand (1991) characterized additional lower molecular weight polypeptides consistent with the Western blotting data reported here. However, caution must be exercised when making a direct comparison, since they obtained E/S parasite material by *in vitro* culture for 9 h only and used serum from schistosome-infected humans to immunoprecipitate antigens. Furthermore, they concluded that immunogens released by adult worms were not associated with the tegumental membrane. In contrast, our results show that 4 immunodominant molecules (M_r 24, 19, 18 and 14 kDa) were present in the tegumental membrane preparation. One suggestion for the observed differences could be that incorporation of radio-isotope label *in vitro* limits the true extent and nature of protein release by worms, since it follows that the identification of antigens is restricted to those that contain methionine (Lewis & Strand, 1991) or leucine (Atkinson & Atkinson, 1982); proteins in which these amino acids are scarce would not be readily demonstrable by immunoprecipitation.

The secretory nature of the antigens was confirmed by determining their site of release from the tissues of adult worms using 2 different experimental approaches. The first technique involved generation of a series of soluble protein fractions from whole worms, worm segments, tegument membranes and 3 h culture supernatants. Weak antibody reactivity was demonstrated against 32, 30, 24 and 19 kDa proteins isolated from schistosome tegumental membrane, and hence it is suggested that these proteins

originate from the membrane of adult worms. It is plausible that an enzymatic mechanism is responsible for surface molecule release, as described by Pearce & Sher (1989). The data support the view that the tegument membrane of adult worms represents an immunologically active site and that some of its products are shed into the host bloodstream during infection. The pattern of proteins detected in the culture supernatant was analogous to that in the tegument membranes. This indicates that most of the immunogenic products released by schistosomes, cultured for a short period *in vitro*, originate from the tegument, a finding supported by Rotmans *et al*. (1981) who showed that the gut contributed very little. Furthermore, following a pulse-chase incubation with adult worms, Wilson & Barnes (1979) showed evidence for the more prolific secretion of exportable leucine-containing protein from the tegument (67–80 %), compared to the gut (20–33 %). Hence, it is plausible that the 3 h incubation of schistosomes *in vitro* restricts the identification of antigens to more abundant products released from the tegument. The implication is that *in vitro* maintenance of worms limits their secretory potential by either, discouraging them from opening their oesophagous to the less favourable environment, or because the adults no longer feed actively and therefore are less likely to release the products of digestion (Rotmans & Burgers, 1987). Additional sources of released material were not identified by Western blotting, with the exception of the oesophageal preparation, in which a 28 kDa antigen was detected. An oesophageal-derived molecule of similar M_r, possessing anticoagulant properties, has been partially cloned by Dr Agnew and co-workers (personal communication). This protein is present in the posterior region of the oesophagous, within the cells of the oesophageal gland. At a subcellular level it appears to be contained within secretory vesicles, indicating that it is released into the narrow lumen of the oesophagous.

Immunolocalization of proteins within worm tissue was used to confirm the cellular source(s) of released antigen. Antibody to the 67 and 53 kDa antigens was evident as packets of intense reactivity in the gastrodermis, indicating that the antigens are synthesized and stored within vesicles ready for transport to the gut lumen. In comparison, the subcellular source of the 38 and 32 kDa antigens in the gut epithelium was less distinct; unlike the 67 and 53 kDa proteins their inclusion in secretory vesicles was debatable since reactivity was not aggregated into foci of fluorescence. Therefore, the 38 and 32 kDa products may represent cytoplasmic components not involved directly in the process of digestion. Alternatively, they may be generated in such abundance that the limited resolving power of the light microscope cannot distinguish individual pockets of reactivity. It is noteworthy that the proposed haemoglobinase, Sm32 (Deelder *et al*. 1977), also displayed the same cellular distribution pattern as the 38 and 32 kDa proteins described in the present study (Chappell & Dresden, 1988). In contrast, eluted antibody to the 24, 19, 18 and 14 kDa proteins was immunolocalized to the tegument, sunken subtegumental cell bodies and the zone in between. The dorsal regions of male worms exhibited more intense fluorescent staining compared to the ventral surface. In view of this observation, it is worth emphasizing that the tubercules of the male parasite allow it to gain purchase along the mesenteric veins. Thus, it is plausible that the dorsal surface is continually exposed to mechanical wear during the parasite's residence in the host bloodstream, thereby establishing it as a richer source of released antigens than the worm's ventral region. The enhanced binding of antibody to this site appears to validate the supposition. Furthermore, the pattern of tegumental staining is consistent with the turnover of constituents, as described by other workers. Membraneous vesicles, pre-synthesized in the subtegumental cell bodies are translocated to the surface of adult worms, via cytoplasmic connections, where they participate in the process of membrane replacement (Hockley & McLaren, 1973; Wilson & Barnes, 1974*a*). The cell bodies, cytoplasmic tubules (which run between the cell bodies and the tegument) and the antigenically dense tegumental layers were all clearly differentiated in this study. The characteristic honeycomb appearance was probably due to the negative reaction of the longitudinal muscle fibre cytoplasm (Riengrojpitak *et al*. 1989). The lack of reactivity against other cell membranes by antibody to 24, 19, 18 and 14 kDa antigens suggests that these components are specific to the tegument. However, a certain degree of cross-reactivity must exist, since unfractionated WTS reacted with all cell types, including non-secretory tissues (parenchymal cells). Furthermore, a polarization of staining was observed in the tegument, from the inner basal membrane to the apical membrane, which probably reflects the accumulation at higher densities of protein transported through the syncitial cell layer.

In conclusion, the culture of adult parasites *in vivo* has facilitated the analysis of worm proteins released during a schistosome infection. This technique has many advantages over *in vitro* methods of culture since (1) the antibody response is used as a sensitive detector of scarce released products, (2) the transfer of adult male worms avoids egg-induced pathology, thereby permitting the study of E/S material over an extended time-course, (3) the host's bloodstream provides an ideal environment for parasite maintenance, (4) the release of significant amounts of somatic contaminants from dead or dying parasites is precluded. Notably, following the transfer of adult worms the first antibodies detectable 2 weeks post-

infection were mostly directed against the gut epithelium. As the time-course progressed additional reactivity was expressed against the tegumental membrane. The early response to gut-associated antigens in comparison to tegumental antigens might be the result of very immunodominant epitopes on the gut-derived products, or of the relatively large amounts of material released into the host circulation rapidly, post-transfer. By comparison with *in vivo* culture, tegumental constituents are the most abundant released products *in vitro*, which suggests that short-term culture promotes a biased release of antigens due to inhibition of normal parasite function. The study also confirms the tegument as a significant source of released antigens, in spite of its primary role as a site for evasion of the host immune response.

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